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A METHOD FOR THE ESTIMATION OF MILK LIPASE

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A METHOD FOR THE ESTIMATION OF MILK LIPASE

by

Lloyd Muir Smith, B.Sc.

A thesis submitted from the Department of Dairying in partial fulfillment of the requirements for the degree of Master of Science at the University of Alberta.

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A METHOD FOR THE ESTIMATION OF MILK LIPASE

INTRODUCTION

GENERAL

In recent years a flavor defect of dairy products known as "Rancidity" has been receiving an increasing amount of attention. The main dairy products susceptible to this type of undesirable flavor are milk, cream, butter, whole milk powder, sweetened condensed milk and raw milk cheddar cheese. In the Province of Alberta during certain seasons of the year as high as 20% of the cream received at creameries exhibits this defect. The problem was referred to the University of Alberta Department of Dairying in 1943 because of the resulting loss to the cream producers and the lowering of butter quality. Since that time joint research on various aspects of rancidity has been carried on with the Department of Animal Science. The work reported in this thesis was undertaken to develop a method for the determination of the lipase content of or lipolytic activity in milk. It was also hoped that such a method might serve as a basis for modified methods for the determination of lipase and for the study of lipolysis in other dairy products and bacterial cultures.

THE NATURE OF RANCIDITY

The deterioration of fats involving the development of undesirable flavors is an important problem in the food industries in general. While this deterioration may involve both hydrolysis and oxidation, it has become customary for the public and many food chemists to refer to off-flavors in fats as a rancid flavor or to describe the condition as rancidity. In the dairy industry it is customary to reserve the term "rancid" for designation of the off-flavor caused by free fatty acids resulting from hydrolysis, and to designate the off-flavors resulting from oxidative changes "oxidized flavor" or "tallowy."

Milk fat differs from other naturally occurring fats in its high content of volatile fatty acids, especially butyric acid which does not occur in significant amounts in any other fat. Rancidity in dairy products is caused by hydrolysis of the milk fat with the release of free fatty acids which impart to the milk their characteristic unpleasant odors and flavors. This hydrolysis or lipolysis is brought about by the enzyme lipase. As some of the lower fatty acids in milk fat, particularly butyric acid, have powerful and disagreeable odors, the presence of very small amounts of these acids may result in a rancid odor and flavor.

The intensity of rancidity in milk is proportional to the amount of free fatty acids present and the terms used in

describing the flavor may vary from "unclean", "cowy" and "goaty" to "bitter" or "rancid." It has been observed that rancid milk not only exhibits a disagreeable odor and a sharp, acrid or bitter taste, but also produces a peculiar throat irritation. Dunkley and Wood (1945) have suggested that the fatty acids of low molecular weight (butyric, caproic and caprylic) are responsible for the characteristic aroma, sharp taste and throat irritation, while the acids of higher molecular weight (capric and lauric) produce the bitter taste.

In general, two types of rancidity caused by lipolysis of milk fat are now recognized. They are known as spontaneous rancidity and activated rancidity. Spontaneous lipolysis is the type which develops in milk without any special treatments other than cooling. In induced lipolysis the system requires some special treatment or "activation" such as homogenization, vigorous agitation or a special temperature treatment. Such "activation" procedures may change the normal surface of the milk fat globules and in some manner make the fat more susceptible to lipolysis.

Wide differences are observed in the susceptibility of milk to lipolysis or the development of the rancid defect. To explain such differences and if possible prevent milk from going rancid, two main problems concerning lipase have been studied by investigators:

(1) the factors that determine the initial amount of lipase secreted in the milk.

(2) the factors that influence the activity of the lipase secreted or the susceptibility of milk fat to lipolysis.

It has been suggested that the period of lactation, the season of the year, the type of feed and other factors may affect the amount of lipase in milk. However, investigators are not in agreement on this phase of the problem. For example, while most workers agree that stage of lactation does influence rancidity, they are not agreed on the explanation. Palmer (1922), Hileman and Courtney (1935) and others have found that the amount of lipase tends to increase towards the end of lactation; while Herrington and Krukovsky (1939) and Mattick and Kay (1938) and others have found little or no such correlation.

Dunkley (1946) has suggested that the increased susceptibility to rancidity of certain milks produced in late lactation may be due to a change in the activity of the lipase caused by a change in the action of an enzyme inhibitor or activator, or that the butterfat has been altered in such a way as to favor more rapid lipolysis. Relatively large amounts of lipase may be present, but unless conditions are favorable for its activity it does not break down the fat.

Castell (1942) has pointed out that many of the differences and contradictory statements in the literature will disappear when more consideration is given to differentiating between the amount of lipase present and the conditions which intensify

its activity. It is for the above reason that Castell defines rancidity as "the product of lipase together with those particular conditions which permit the enzyme to function."

THE NATURE OF LIPASE

Milk normally contains lipase which is associated with the serum and the enzyme is believed to have at least two functions. Under certain conditions it may be involved in the synthesis of fat while under other conditions it may reverse this role the fat being hydrolyzed to form free fatty acids and glycerol. Lipase is produced by the living cell and is found not only in milk but in the liver, pancreas and blood serum of the body as well as in many plants, yeasts, moulds and bacteria. There are significant differences in the properties of lipases from different parts of the body or from different plants or micro-organisms.

Very little is definitely known about the chemical constitution of the lipase or lipases in milk. The enzyme is at least partly protein in nature. The amount of lipase in milk may be very small and it has not been isolated as a pure substance. Since very few enzymes have been isolated, it has been customary to express their concentration in terms of their activity. The study of the lipase content of dairy products is complicated by various factors that influence the activity of the lipase that may be present. For example, Dorner and Widmer (1931) found that homogenization of raw milk greatly increased subsequent lipolysis. It has been shown by Krukovsky and Sharp (1938) that lipase may be activated by shaking, particularly when the fat is in the liquid

state. Herrington and Krukovsky (1939) found that lipolysis in cold milk is accelerated by warming the milk slightly and recooling. Several other factors such as pH, dissolved oxygen, traces of heavy metals, oxidizing and reducing agents and the presence of certain bacteria are believed to affect the activation of lipase. The lipolytic activity of lipase seems to be lost on proteolysis. Hylynka and Hood (1942) suggest that the tendency to produce a rancid flavor in cheddar cheese is greatly decreased by increasing the proteolytic enzymes, pepsin or rennin, in making the cheese. Hetrick and Tracy (1948) have shown that the lipase of milk is inactivated by heat and this fact is of great practical significance.

It has been suggested that there is more than one lipase in milk. Dorner and Widmer reported the existence of two lipases in milk, one heat resistant to some degree and the other more heat labile. Herrington and Krukovsky found two lipases, one inhibited completely by small amounts of formalin and the other apparently resistant to moderate amounts of formalin. Gould and Trout (1936) showed that there may be a difference in lipolysis of homogenized and non-homogenized raw milk. Lipolysis in homogenized milk was not reduced by formalin or by traces of copper and this might be considered as evidence of two lipases. Peterson, Johnson and Price (1943) investigated the stability of the lipase system over a 12 hour

period at 0°C and concluded that two enzymes could be present and that the ratio of the two seemed to vary from cow to cow.

HISTORICAL

Rice and Markley (1922) and Hileman and Courtney reviewed the early methods for the determination of lipase. According to Rice and Markley, Marfan (1901) was probably the first to report that experimental proof had been obtained of the presence of a lipolytic enzyme which splits monobutyrin in the milk of man and the cow.

Between 1901 and 1909 several reports of investigations on the presence of lipolytic enzymes in milk and milk products appeared in the literature and are referred to by Rice and Markley. A great variety of substrates including simple esters as well as butterfat, cottonseed oil, castor oil, olive oil and almond oil were used. Preservatives such as formaldehyde and chloroform were used to inhibit bacterial growth in the samples but they also retarded lipase activity. The results reported were conflicting and the question of the presence of lipase in milk was not satisfactorily answered.

Maass (1909) presented evidence that cow's milk contained a true lipase capable of hydrolyzing milk fat. Maass used formalin as a preservative and sterilized cream as a substrate. He found that the addition of raw milk or cream caused an increase in titratable acidity after incubation at 35--38°C and that this increase was proportional to the amount of inoculum. This is characteristic of an enzymatic rather than of a bacterial process. Maass also reported that the amount

of enzyme present in milk increases at the end of the lactation period.

In 1922, Rice and Markley confirmed the report that normal milk does contain an active lipase which sets fatty acids free from milk fat in the absence of bacterial growth. Boiled cream saturated with cane sugar was used as the substrate for lipase and the extent of lipolysis after incubation from three to thirty days was measured by titrating the cream with standard alkali to the phenolphthalein end point.

Reder (1938) made a chemical examination of rancid milk and found it to have a slightly higher chloride and lower lactose content and a higher concentration of total solids than normal milk. Besides the development of a rancid odor and flavor, several other changes occur in milk as a result of lipase action. Most studies of lipase action in milk have been based upon measurements of the increase in titratable acidity of the milk, but several investigators have used changes in pH, fat acidity or surface tension as a measure of lipolysis.

Herrington and Krukovsky, Hylynka and Hood, and Gould and Trout all measured lipase activity by titrating the free acid developed in the fat phase of milk. The milk was held at a low temperature for periods of a day or more. The samples were then churned, the melted butterfat centrifuged and an aliquot of the clear fat titrated with a standard alkali.

Tarassuk (1939) found that, as a result of lipase action, one of the most profound changes that occurs in milk is the lowering of its surface tension. This lowering of the surface tension is due to the presence of free fatty acids liberated by the hydrolysis of the fat. As the hydrolytic rancidity develops, the surface tension of milk decreases from a normal value of 49-51 to 39-40 dynes per centimeter. However, many factors other than lipolysis influence the surface tension of milk. Dunkley found that care must be taken to standardize the procedure for collection and treatment of the samples in order to minimize the effects of various factors which influence the surface tension. Hetrick and Tracy (1948) used surface tension changes supplemented with organoleptic examination as a measure of lipase activity in their studies on milk lipase inactivation by heat.

Reder estimated lipase by measurement of the amount of hydrolysis of tributyrin added to raw whole milk. Formaldehyde was used to inhibit bacterial growth during the incubation period of twenty-four hours. The pH of the reaction mixture was not controlled.

In 1938, Mattick and Kay questioned the reliability of many of the earlier methods for the determination of milk lipase. Among the procedures which these investigators considered dubious were the following:

"(1) The incubation of admittedly non-sterile material suspected of containing a lipase with fat for long periods of

time, frequently under conditions almost optimal for bacterial reproduction.

(2) The estimation of lipolysis by titration of increase in the total acidity of a reaction mixture, where processes other than lipolysis, as possible sources of the increase in acidity, had not been excluded.

(3) Reliance on antiseptics not previously proved to be efficient.

(4) Use of antiseptics which seriously inhibit lipase action.

(5) Use of substrates other than triglycerides for the estimation of lipolytic activity."

Mattick and Kay devised an improved method for estimating lipase, or tributyrinase as they prefer to name the enzyme responsible for splitting tributyrin. It consisted in the titration of butyric acid, formed under standard conditions of time, temperature, acidity and volume, from the action of the milk enzyme on tributyrin in a buffer solution.

Milk was buffered at pH 8.5 using sodium di-ethyl barbiturate, tributyrin was added as a substrate and the reaction mixture held at 37°C with gentle shaking. Aliquot portions were removed at the start and after various incubation times up to six hours. The aliquot portions were acidified with phosphoric acid, steam distilled to recover the free volatile acids and the distillates titrated using phenolphthalein as

indicator. The hydrolysis of the tributyrin was determined by the titer of the steam distillate and was considered to be proportional to the tributyrinase present. Increases in bacterial population during incubation were small and could be considered a negligible source of error in the determination.

In 1943, Peterson, Johnson and Price suggested a method for the determination of milk lipase. This method resembled that of Mattick and Kay except that the hydrolysis of tributyrin was measured by removing aliquot portions of the reaction mixture at the beginning and at the end of the incubation period and titrating them in the presence of alcohol and ether to the thymolphthalein end point. The shortening of the incubation time to thirty minutes and the elimination of the time-consuming steam distillation required in the Mattick and Kay method made this procedure more readily adapted to routine determinations. Peterson, Johnson and Price (1948) have recently reported a method for the determination of cheese lipase. This procedure is similar in principle to their milk lipase determination.

Kelly (1944) (1945) developed a method of estimation of milk lipase in which the milk to be tested is first dried and then extracted with acetone and ether. A soft fine precipitate was obtained which would keep indefinitely. Various substrates including tributyrin and sterilized butter oil were employed. Some samples were immediately extracted with ether to serve as blanks, the remainder were incubated at 37°C, usually for twenty-four hours, before extraction. Alcohol was added to

the extract which was titrated against standard alkali using phenolphthalein as indicator. Kelly pointed out that one disadvantage of his method is that the physical condition of the samples is not the same as it would be in undried milk.

Johnson and Gould (1946) studied the variation in the acid degree of milk fat as affected by churning and extraction procedures. In their extraction procedure, rancid cream was shaken with a mixture of ethyl ether and Skellysolve, the solvents being in the ratio of 1:1. The mixture was centrifuged, the supernatant ethereal portion removed by syphon and the solvent evaporated. The degree of fat splitting resulting from lipase activity was determined by titration with 0.1N NaOH to the phenolphthalein end point.

Lubert (1949) reviewed methods for the detection of lipolytic micro-organisms and for the study of lipolytic activity in bacterial cultures. Using a method based on the procedure proposed for milk lipase in this paper, he studied the lipolytic activity in cultures of various micro-organisms, with particular reference to Pseudomonas fluorescens, and in cheddar cheese.

PRELIMINARY INVESTIGATIONS

In order to study the properties of lipases which may be present in dairy products, a reliable and yet versatile method for the determination of the lipase content of fluids such as milk and bacterial media was needed. The method should be simple enough that it can be applied routinely to large numbers of samples.

Dunkley selected the Peterson, Johnson and Price technique and applied it to milk lipase determinations. However, Dunkley was not able to obtain satisfactory reproducibility in his results. The author also applied this method to routine lipase determinations of milk and found it unsatisfactory.

The main difficulty is in determining the end point of the titration. Since the solution which is titrated contains milk salts and proteins and, in addition, is buffered at pH 8.5, it has appreciable buffer capacity at the titration end point and does not give a sharp color change.

As a result of numerous trials, Dunkley devised a tentative procedure based on that of Peterson, Johnson and Price. The necessity for titrating the liberated butyric acid in the presence of proteins and barbiturate buffer was eliminated by acidifying the reaction mixture with phosphoric acid, extracting it with ether and titrating an aliquot of the ether extract. Dunkley conducted experiments to determine the reliability of the method and the importance of standardi-

zing the conditions under which it was carried out so that the measure of lipolysis could be considered directly proportional to the lipase content. Lipase determinations were made routinely at weekly intervals on a selected group of cows in the University herd. On the basis of this work, Dunkley suggested certain modifications to improve the tentative procedure. The author has investigated the feasibility of using these modifications and has carried out further work on the reliability of the method finally adopted.

EXPERIMENTAL

I. METHOD

Reagents

Borate Buffer - 6.23g boric acid and 50 ml Normal NaOH made up to 1 liter.

Tributyrin - obtained from the Eastman Kodak Company, Rochester, N.Y.

Molar Phosphoric Acid

Ethyl Ether

Titration Mixture - containing 1 liter 95% ethanol, 1 liter distilled water, 50 ml 0.1% alcoholic Cresol Red. A few drops of 0.05N NaOH are added to give the mixture a pH of 7.4.

Standard 0.025N NaOH.

Procedure

The sample of milk to be tested is centrifuged and the cream layer removed by means of a spatula. Two ml of the skim milk are pipetted into a test tube containing 5 ml of the borate buffer. The mixture is adjusted, if necessary, to pH 8.8 (at 22°C) using M/10 NaOH or M/10 phosphoric acid. Fresh normal milk samples seldom require any adjustment.

The buffered milk is placed in a water bath at 37°C and allowed to reach this temperature in 3 minutes. Then 0.6 ml tributyrin are added, a rubber stopper inserted, and the mixture is shaken vigorously for 5 seconds to emulsify the tributyrin. The reaction mixture is then returned to

the water bath and the rubber stopper loosened. The sample is not agitated during the reaction period.

At the end of the reaction period (30 minutes after the addition of the tributyrin), 5 ml of M phosphoric acid are added, the test tube is inverted twice and transferred to a 20°C water bath. After allowing 3 minutes for the mixture to reach this temperature, 10 ml of ethyl ether are added and the tube is shaken vigorously for 5 seconds. The test tube is then returned to the 20°C water bath. The ether layer usually separates rapidly after the extraction but approximately 5 minutes should be allowed to permit small water droplets to settle out of the ether layer. The rubber stopper should be loosened 2 or 3 minutes before a 5 ml aliquot of the clear ether extract is transferred by means of a pipette to a 100 ml beaker containing 10 ml of the titration mixture. The ether extract and titration mixture are then titrated with the standard NaOH to a faint orange color using a burette calibrated at 0.01 ml intervals. The number of ml of 0.025N NaOH required to reach this end point is designated as the "Titer."

The blank determination is carried out in the same manner except no time is allowed for hydrolysis of the tributyrin to take place. The M phosphoric acid is added immediately after the samples have been shaken with the tributyrin. The number of ml of 0.025N NaOH required to

titrate the blank determination is reported as the "Blank."

The difference in ml between the Titer and the Blank represents tributyrin hydrolysis and is referred to in this paper as "Hydrolysis."

II. RELIABILITY OF EXTRACTION PROCEDURE

(a) Titration of the Ether Extract.

The procedure described under "Method" involves the titration of ether extracts of butyric acid in the presence of low concentrations of boric and phosphoric acids.

Since the dissociation constants and the end points of indicators as determined in aqueous solutions would not apply to the above titration which is carried out in the presence of ether, potentiometric titrations were made to determine the end point desired and the suitability of the indicator for this titration. The following solutions of the acids involved in the lipase determination were prepared in approximately the concentrations in which they would be present in the reaction mixture at the time it is extracted with ether:

0.008 M butyric acid
0.005 M butyric acid
0.5 M phosphoric acid
0.05 M boric acid.

Ten ml of each solution were extracted with 10 ml of ether. A 5 ml aliquot of each ether extract was added to 10 ml of titration mixture and the solutions were titrated potentiometrically with 0.025N NaOH using a Beckman glass electrode pH meter. The meter was standardized at pH 8.60 and was checked with the standard buffer solution before each titration. The end point pH reading was taken at the

first change to a faint orange color. The apparent pH readings obtained were slightly high because of the influence of the ether, but it is only necessary to consider changes in pH (not absolute pH values) to establish the reliability of the titration.

Figure 1 shows the curves obtained for:

A - 0.008M butyric acid
B - 0.005M butyric acid
C - 0.5M phosphoric acid
D - 0.05M boric acid
E - distilled water.

The end point color change occurred between pH 8.55 and 8.60 in these titrations. From the above curves it is apparent that butyric acid is easily extracted, can be readily titrated and gives a sharp end point when Cresol Red is used as the indicator. The presence of low concentrations of boric and phosphoric acids does not interfere with the titration of the butyric acid.

(b) Extraction of Free Butyric Acid.

Replicate test tubes each containing 2 ml of pasteurized skim milk buffered with 5 ml of borate buffer were prepared. After 0.6 ml of tributyrin were added, each sample was shaken for 5 seconds. Then increasing amounts of 0.05 N butyric acid were added to duplicate tubes. Each sample was acidified by adding 3.5 ml of 1.5M phosphoric acid, inverted twice, and then extracted with 10 ml of ether. A 5 ml aliquot of the ether extract was titrated with 0.025N NaOH as in the regular procedure. The data are given in Table I.

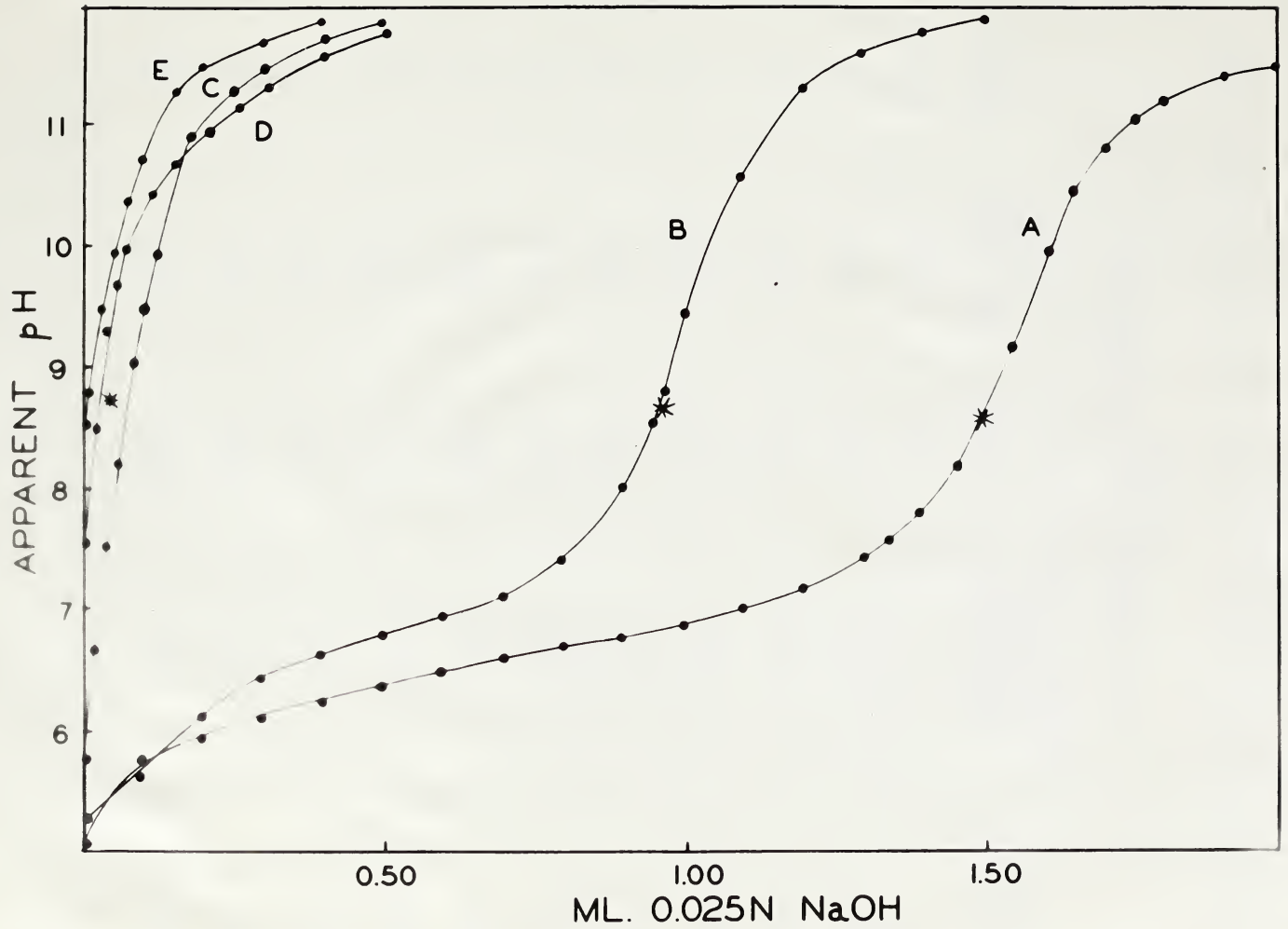


Figure 1 - Potentiometric Titration Curves

TABLE I

EXTRACTION OF FREE BUTYRIC ACID

Sample Number	0.05N Butyric Acid, Ml	Titer	Average Titer Corrected For Blank	Percent Extraction
Blank	0.00	0.17	--	--
Blank	0.00	0.17		
1	0.20	0.35	0.18	90%
1	0.20	0.34		
2	0.40	0.53	0.37	92%
2	0.40	0.55		
3	0.60	0.72	0.54	90%
3	0.60	0.70		
4	0.80	0.91	0.74	92%
4	0.80	0.91		
5	1.00	1.09	0.92	92%
5	1.00	1.09		
6	1.20	1.28	1.11	92%
6	1.20	1.27		
7	1.40	1.45	1.29	92%
7	1.40	1.46		
8	1.60	1.66	1.47	92%
8	1.60	1.61		
9	1.80	1.81	1.66	92%
9	1.80	1.86		
10	2.00	2.00	1.83	91%
10	2.00	1.99		

Figure 2 shows that there was a direct relation between the concentration of free butyric acid in the sample and the amount extracted by the ether as measured by the titer corrected for the blank determination. The percentage recovery of the added butyric acid was around 90 percent.

(c) Varying the Agitation during Extraction.

Twenty ml of pasteurized skim milk were buffered with 50 ml of borate buffer and then 10 ml of approximately 0.05N butyric acid were added. A series of 8 ml samples of this mixture was pipetted into test tubes. The samples were brought up to 37°C, 0.6 ml tributyrin added and the tubes shaken for 5 seconds. After a 30 minute reaction period at 37°C, the samples were acidified with 4 ml of M phosphoric acid. Ten ml of ether were used to extract the free butyric acid but the amount of shaking was varied. A set of blanks without butyric acid was also prepared and, after acidification with 5 ml of M phosphoric acid, was extracted with ether in a similar manner.

The data given in Table II demonstrate that shaking each sample with the ether approximately 25 times in 5 seconds is sufficient agitation to satisfactorily extract the free butyric acid present.

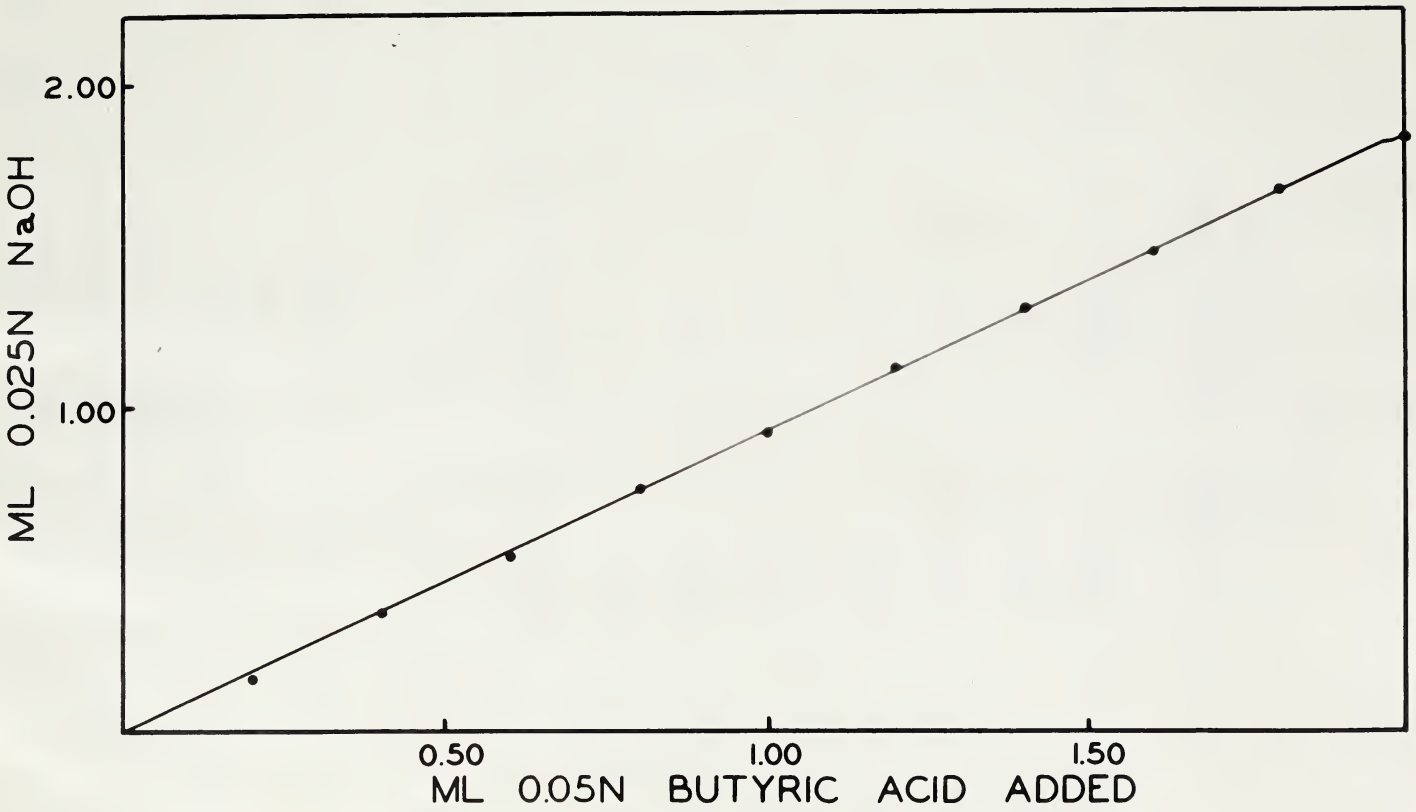


Figure 2 - Extraction of Butyric Acid

TABLE II

VARYING THE AGITATION DURING

ETHER EXTRACTION OF BUTYRIC ACID

Sample Number	Times Shaken	Blank	Titer
1	5	0.15	1.00
1	5	0.13	0.95
2	10	0.16	1.02
2	10	0.13	1.03
3	20	0.13	1.06
3	20	0.15	1.04
4	30	0.15	1.05
4	30	0.14	1.03
5	40	0.14	1.05
5	40	0.14	1.04

(d) Varying the Temperature of Extraction.

A series of samples was prepared by adding 2 ml of pasteurized skim milk* to 5 ml of borate buffer in each test tube. One ml of 0.05N butyric acid was then pipetted into each sample tube. After the addition of 0.6 ml of tributyrin, the samples were shaken for 5 seconds and 5 ml of M phosphoric acid were added to each test tube. Triplicate samples were then placed in water baths held at 15°, 20°, 25° and 30°C. After allowing the samples to come to the required temperature, each triplicate set was extracted with ether which had been

*Skim milk held at 80°C for 5 minutes and then cooled quickly to 25°C.

held at the same temperature and the determinations were completed in the usual manner.

TABLE III

VARYING THE TEMPERATURE

OF ETHER EXTRACTION OF BUTYRIC ACID

Sample Number	Temperature of Extraction °C	Titer
1	15	1.06
1	15	1.05
1	15	1.07
2	20	1.02
2	20	1.05
2	20	1.05
3	25	1.07
3	25	1.05
3	25	1.07
4	30	1.06
4	30	1.06
4	30	1.05

The titers shown in Table III demonstrate that variations in temperature of extraction within the range 15°C to 30°C have no significant effect on the amount of butyric acid extracted.

(e) Varying the Concentration of Tributyrin.

Forty ml of pasteurized skim milk* were buffered with

*Skim milk held at 80°C for 5 minutes and then cooled quickly to 25°C.

100 ml of borate buffer and then 20 ml of 0.05N butyric acid were added. Eight ml amounts of this mixture were pipetted into test tubes and placed in a water bath at 37°C. About 3 minutes later increasing concentrations of tributyrin were added, the samples shaken for 5 seconds and then held at 37°C for 30 minutes. The blanks were prepared by adding 2 ml of the pasteurized skim milk to 5 ml of borate buffer and shaking as before with the appropriate amount of tributyrin. After the 30 minute holding period at 37°C, 3.5 ml of 1.5M phosphoric acid were added to all the test tubes and the determinations completed as in the regular procedure.

Table IV shows that as the amount of tributyrin is increased up to 1.50 ml, the titers obtained increase slightly. When the titers are corrected for the blank values, the addition of varying amounts of tributyrin has no appreciable effect on the amount of butyric acid extracted by the ether. However, as the tributyrin concentration is increased, less ether separates out as a clear layer and greater care is necessary in pipetting the 5 ml aliquot for titration.

TABLE IV

EFFECT OF VARYING THE CONCENTRATION OF TRIBUTYRIN ON ETHER EXTRACTION OF BUTYRIC ACID

Sample Number	Tributyrin Added, Ml	0.05N Butyric Acid, Ml	Titer	Blank	Corrected Titer
1	0.00	1.00	0.97	0.10	0.87
2	0.10	"	1.03		0.92
3	0.20	"	1.04	0.12	0.92
4	0.30	"	1.06		0.93
5	0.40	"	1.06	0.14	0.92
6	0.50	"	1.08		0.91
7	0.60	"	1.07	0.19	0.88
8	0.80	"	1.06		0.86
9	1.00	"	1.11	0.21	0.90
10	1.20	"	1.11		0.88
11	1.50	"	1.16	0.26	0.90

III FACTORS AFFECTING THE BLANK DETERMINATION.

The blank determination may be made in either of two ways:

(a) Carry out the determination as outlined in the procedure given under "Method" but allow no time for hydrolysis of the tributyrin to take place.

(b) Carry out the determination as outlined in the procedure given under "Method" but destroy the enzyme in the milk by heat before adding the tributyrin.

For routine determinations the first procedure proved to be the more convenient and was adopted.

With fresh milk samples the blank has been found to be quite constant at 0.16 ml of 0.025N NaOH (within experimental error of about ± 0.01 ml), and this value can be used as a standard correction for routine determinations. This means that only one titration may be required for each determination. However, this is not possible when dealing with samples which may previously have undergone lipolysis or souring. With such samples the free fatty acids or produced lactic acid increase the value of the blank.

(a) Tributyrin Breakdown.

To determine the extent of tributyrin hydrolysis in the absence of lipase activity during the 30 minute reaction time, a series of buffered pasteurized skim milk samples, each

containing 0.6 ml of tributyrin, was held at 37°C for varying periods of time. The determinations were then completed in the usual manner. It is apparent from the data given in Table V that no appreciable breakdown of tributyrin occurs during the 30 minute reaction period. Therefore, it is not necessary to correct the blank for acidity due to the hydrolysis of tributyrin during the reaction period.

TABLE V

EFFECT OF REACTION TIME AT 37°C

ON TRIBUTYRIN

Number	Tributyrin, Ml	Reaction Time, Min.	Titer
1	0.6	0	0.15
2	"	30	0.15
3	"	60	0.17
4	"	90	0.20

(b) Varying the Concentration of Boric Acid.

In the lipase determination of milk the borate buffer used is approximately 0.1M with respect to boric acid. At the time of extraction the concentration is approximately 0.04M. Increasing amounts of 0.3M boric acid were added to a series of replicate milk samples buffered with the regular

borate buffer. Then 0.6 ml of tributyrin were shaken with each and the samples were treated as blank determinations.

TABLE VI

EFFECT OF VARYING THE CONCENTRATION OF BORIC ACID ON THE BLANK

Number	0.3M Boric Acid, Ml	Tributyrin, Ml	Blank
1	0.00	0.6	0.12
1	0.00	"	0.12
2	0.25	"	0.13
2	0.25	"	0.13
3	0.50	"	0.12
3	0.50	"	0.12
4	0.75	"	0.12
4	0.75	"	0.11
5	1.00	"	0.12
5	1.00	"	0.11

The results given in Table VI demonstrate that an appreciable increase in the amount of boric acid present does not cause a significant variation in the value of the blank. This means that the composition of the borate buffer may be varied when the proposed lipase assay is to be applied to other dairy products or bacterial cultures.

(c) Varying the Concentration of Phosphoric Acid.

Five ml of M phosphoric acid are used in the determination

to lower the pH of the sample to below pH 2 in order to stop hydrolysis and to permit the subsequent extraction of the liberated butyric acid. For some purposes it is desirable to use a smaller amount of 1.5M phosphoric acid. To determine the effect of such variations, different amounts of both M and 1.5M phosphoric acid were used to acidify replicate blank determinations and the samples were then extracted and titrated in the usual manner.

TABLE VII

EFFECT OF VARYING THE CONCENTRATION OF PHOSPHORIC ACID ON THE BLANK.

Sample Number	M Phosphoric Acid, Ml	1.5M Phosphoric Acid, Ml	Blank
1	4.00		0.12
1	4.00		0.12
2	5.00		0.12
2	5.00		0.12
3	6.00		0.13
3	6.00		0.13
4		4.00	0.14
4		4.00	0.14
5		5.00	0.14
5		5.00	0.15
6		6.00	0.15
6		6.00	0.16

Table VII gives the data obtained. Variations of 1 ml in the amount of M phosphoric acid have little effect on the blank. Similarly variations of 1 ml of 1.5M phosphoric acid have no appreciable effect on the corresponding blank.

(d) Varying the Concentration of Tributyrin.

Replicate samples were prepared by adding 2 ml of pasteurized skim milk to 5 ml of borate buffer. The samples were brought up to 37°C in a water bath, increasing amounts of tributyrin added and each test tube shaken for 5 seconds. Five ml of M phosphoric acid were then added and the samples extracted with ether in the usual manner.

TABLE VIII

EFFECT OF VARYING THE CONCENTRATION OF TRIBUTYRIN ON THE BLANK

Number	Tributyrin, Ml	Blank
1	0.00	0.09
2	0.10	0.10
3	0.30	0.12
4	0.60	0.17
5	1.00	0.21
6	1.50	0.25

The results given in Table VIII indicate that as the amount of tributyrin is increased the value of the blank increases. While differences of a small drop or two in the amount of tributyrin added do not materially affect the value of the blank, care should be taken to add the tributyrin as accurately as possible to avoid this source of error.

(e) Varying the Agitation to Emulsify the Tributyrin.

A series of buffered skim milk samples was prepared and 0.6 ml of tributyrin were added to duplicate samples. The duplicate tubes were shaken 5, 10, 20, 30 and 40 times respectively and then immediately acidified with 5 ml of M phosphoric acid. The samples were then extracted with ether and a 5 ml aliquot of the ether extract titrated in the usual manner.

Table IX shows that the above variations in the amount of agitation to emulsify the tributyrin have little effect on the value of the blank. In the proposed lipase assay the samples are shaken approximately 25 times in 5 seconds.

TABLE IX

EFFECT OF VARYING THE AGITATION

TO EMULSIFY THE TRIBUTYRIN ON THE BLANK

Number	Times Shaken	Tributyrin, Ml	Blank
1	5	0.6	0.14
1	5	"	0.13
2	10	"	0.14
2	10	"	0.13
3	20	"	0.14
3	20	"	0.14
4	30	"	0.13
4	30	"	0.15
5	40	"	0.15
5	40	"	Lost

IV. OTHER FACTORS AFFECTING THE METHOD

(a) Tributyrin Concentration.

Several experiments on the effect of the concentration of tributyrin on the hydrolytic activity of milks from selected cows of the University of Alberta herd were carried out. Samples of milk from individual cows were held at 15°C for approximately one hour after milking and then taken to the Department of Dairying where they were held at 4°C from 1 to 24 hours. Forty ml of each milk were added to 100 ml of borate buffer and 7 ml amounts of the mixture pipetted into test tubes. The buffered samples were brought up to 37°C, increasing amounts of tributyrin added, the test tubes shaken 5 seconds and then replaced in the water bath for the 30 minute reaction period. The determinations were completed in the usual manner except that 3.5 ml of 1.5M phosphoric acid were used to stop hydrolysis. Blank determinations with varying concentrations of tributyrin were also made.

The curves obtained for six milks are shown in Figure 3. In the presence of excess substrate, the enzyme followed a zero order reaction under the conditions used in the determination. Very close to maximum hydrolysis was obtained when 0.60 ml of tributyrin were used as substrate except in the experiment on Milk "A". Further investigations were conducted on this particular milk. After holding the sample for approximately 24 hours at 4°C, an experiment similar to

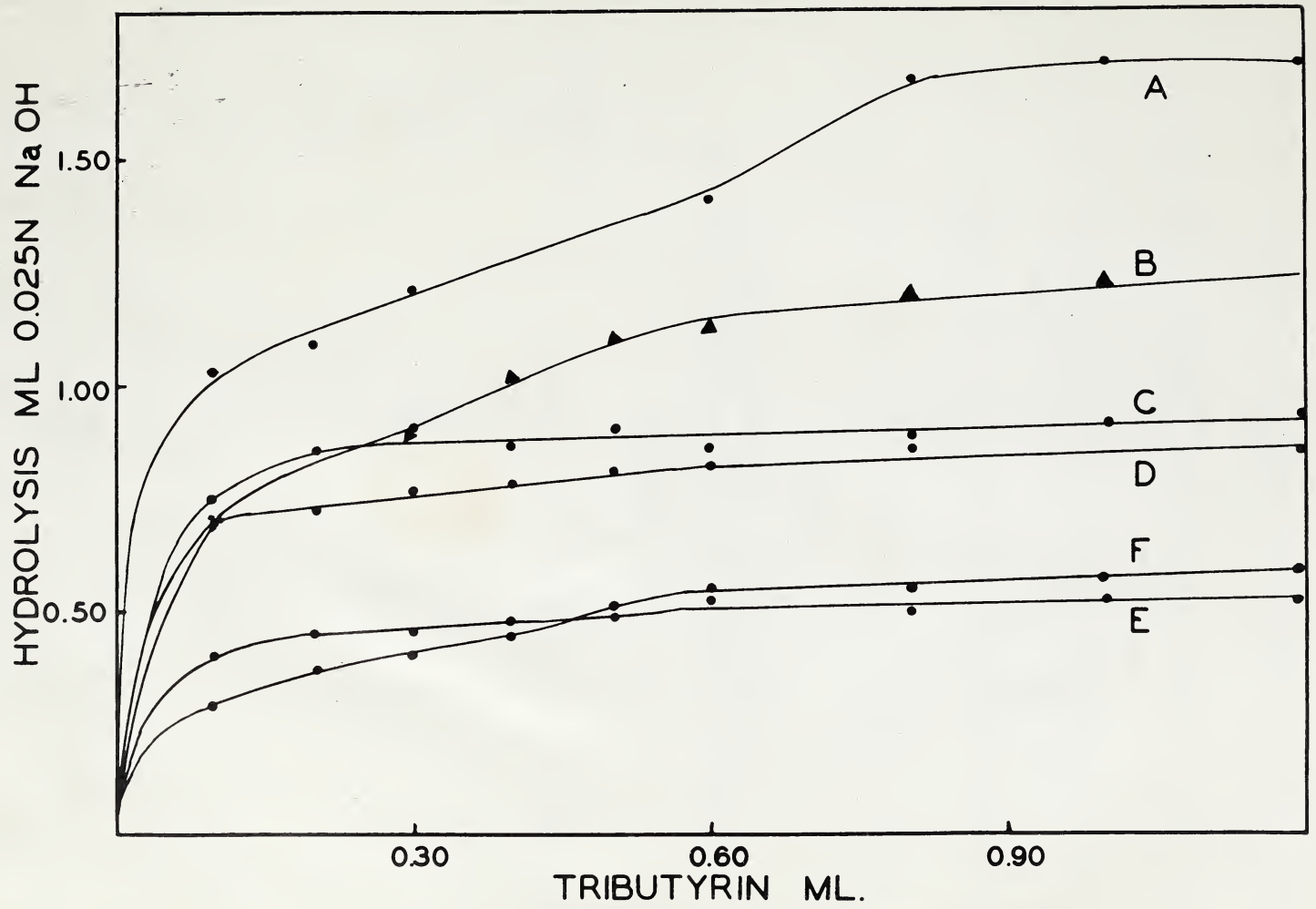


Figure 3 - Effect of Tributyrin on Hydrolysis

that described above was carried out except that the reaction period was increased to 60 minutes. Table X shows that maximum hydrolysis was obtained with 0.60 ml tributyrin under these particular conditions. However, the lipase activity of the milk was possibly reduced by the 24 hour holding period at 4°C. Since it seemed desirable to avoid the use of a 60 minute reaction period for routine work, investigations along this line were discontinued.

TABLE X

EFFECT OF TRIBUTYRIN CONCENTRATION AND 60 MINUTE REACTION PERIOD ON HYDROLYSIS OBTAINED WITH MILK "A"

Tributyrin Ml	Titer	Blank	Hydrolysis
0.00	0.10	0.09	0.01
0.10	1.70	0.14	1.56
0.20	1.86		1.71
0.30	2.10	0.15	1.95
0.40	2.53		2.37
0.50	2.69		2.53
0.60	2.84	0.17	2.67
0.80	2.92		2.73
1.00	Gel	0.21	---
1.20	2.90		2.67
1.50	2.87	0.27	2.60

Other experiments on the effect of the concentration of tributyrin on hydrolysis obtained with both 2 ml and 1 ml amounts of Milk "A" were carried out. The 1 ml samples were prepared by adding 20 ml of milk and 20 ml of distilled water to 100 ml of borate buffer and pipetting 7 ml of the mixture into test tubes. A few drops of 1.5M phosphoric acid were used to adjust the pH to 8.55. Blank determinations with varying concentrations of tributyrin were also made. One hour later the experiment was repeated using 2 ml milk samples prepared by adding 40 ml of milk to 100 ml of buffer. The pH of the buffered milk was again adjusted to 8.55. Curve A1 in Figure 4 shows that 0.60 ml of tributyrin gave maximum hydrolysis when 1 ml of Milk "A" was used.

The great majority of the determinations carried out on the individual milks of cows in the University of Alberta herd have given hydrolysis values under 1.20 ml of 0.025N NaOH. For routine determinations 0.6 ml tributyrin should be satisfactory. However, for milks showing exceptional lipase activity the sample size may be reduced to 1 ml.

Mattick and Kay found that gentle shaking of the reaction mixture during the incubation period slightly increased the hydrolysis of tributyrin. Peterson, Johnson and Price did not agitate their incubation mixture.

A few experiments were conducted to determine the influence of agitation during the reaction period in the proposed lipase assay. Little or no increase in hydrolysis

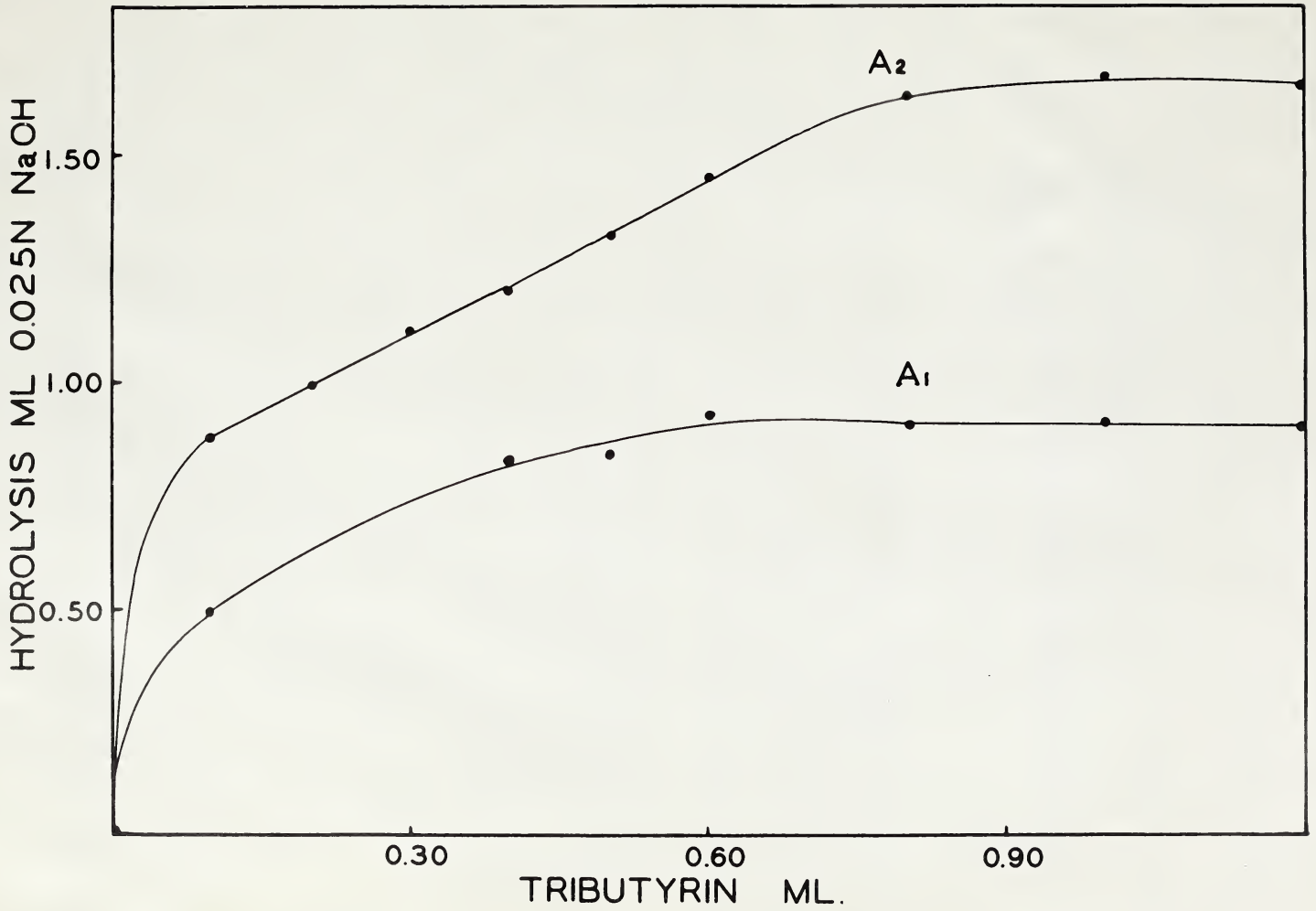


Figure 4 - Effect of Tributyrin on Hydrolysis

was obtained but further work should be carried out before a definite conclusion can be justified. For routine determinations a mechanical means of shaking would be very useful. The possibility that the inclusion of some emulsifying agent, such as lecithin, bile salts or saponin, might help to keep the tributyrin emulsified during the reaction period should also be investigated.

(b) pH

After considerable preliminary work a series of borate buffers was prepared which gave a suitable pH range when 2 ml of milk were added to 5 ml of buffer. Each buffer was 0.1M with respect to boric acid and the composition of the series is given in Table XI.

Five ml of each buffer were accurately pipetted into duplicate test tubes. Two ml of skim milk were then added to one of the duplicate tubes of each buffer and these were brought up to 30°C in a waterbath. After 0.6 ml tributyrin had been added and the mixture shaken 5 seconds, the pH of each sample was determined as quickly as possible. The inside electrodes of a Beckman glass electrode pH meter, previously standardized at pH 8.60 and checked at pH 7.00, were used. No trouble with "drifting" was experienced. The electrodes and electrode cup were rinsed with distilled water at 30°C and then dried with paper tissue between determinations. On completing the pH determinations, 2 ml of the same skim milk

TABLE XI

COMPOSITION OF SODIUM BORATE BUFFERS USED IN DETERMINATION OF OPTIMUM pH FOR HYDROLYSIS OF TRIBUTYRIN

Buffer Number	0.6M Boric Acid Ml	N NaOH Ml	H_2O Ml	Total Vol. Ml
1	16.68	0.00	83.32	100.00
2	"	1.00	82.32	"
3	"	2.00	81.32	"
4	"	2.80	80.52	"
5	"	3.60	79.72	"
6	"	4.00	79.32	"
7	"	4.40	78.92	"
8	"	4.80	78.52	"
9	"	5.20	78.12	"
10	"	5.60	77.72	"
11	"	6.00	77.32	"
12	"	6.40	76.92	"
13	"	7.00	76.32	"
14	"	8.00	75.32	"
15	"	9.00	74.32	"
16	"	10.00	73.32	"
17	"	15.00	68.32	"

TABLE XII

EFFECT OF INITIAL pH ON THE

HYDROLYSIS OF TRIBUTYRIN

	Buffer Number	pH at 30°C	Titer	Blank	Hydrolysis
Milk A	1	6.58	0.27	0.16	0.11
	2	7.44	0.55		0.39
	3	7.94	0.85		0.69
	4	8.18	1.00		0.84
	5	8.39	1.17	0.17	1.00
	6	8.47	1.17		1.00
	7	8.54	1.30		1.13
	8	8.60	Lost		-
	9	8.68	1.34		1.17
	10	8.76	1.42	0.17	1.25
	11	8.84	1.44		1.27
	12	8.92	1.36		1.19
	13	9.00	1.31		1.14
	14	9.23	1.15		0.98
	15	9.45	0.81		0.64
	16	9.66	0.67	0.18	0.49
	17	11.26	2.35		2.17
Milk B	1	6.68	0.25	0.16	0.09
	2	7.61	0.47		0.31
	3	8.04	0.65		0.49
	4	8.30	0.74		0.58
	5	8.49	0.80	0.16	0.64
	6	8.57	0.84		0.68
	7	8.63	0.85		0.69
	8	8.75	0.85		0.69
	9	8.81	0.85		0.69
	10	8.89	0.84	0.16	0.68
	11	8.93	0.82		0.66
	12	9.01	0.82		0.66
	13	9.13	0.78		0.52
	14	9.34	0.65		0.49
	15	9.54	0.51		0.35
	16	9.77	0.41	0.16	0.25
	17	11.38	2.59		2.43

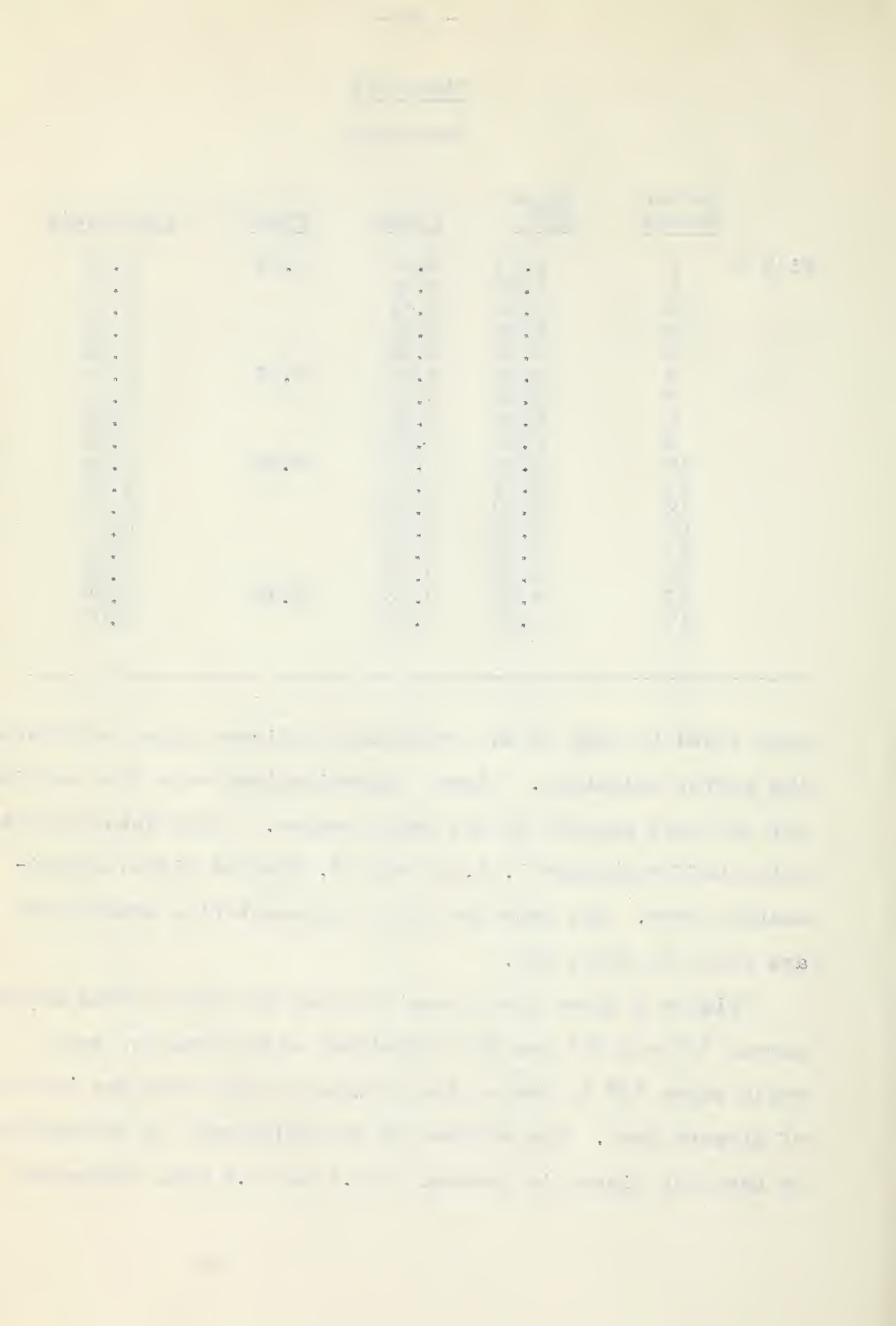
TABLE XII

Continued

	Buffer Number	pH at 30°C	Titer	Blank	Hydrolysis
Milk C	1	6.74	0.24	0.16	0.08
	2	7.58	0.42		0.26
	3	8.01	0.51		0.35
	4	8.25	0.63		0.47
	5	8.43	0.68		0.52
	6	8.52	0.72	0.16	0.56
	7	8.60	0.77		0.61
	8	8.67	0.78		0.62
	9	8.73	0.82		0.66
	10	8.85	0.79	0.15	0.64
	11	8.91	0.75		0.60
	12	8.99	0.72		0.57
	13	9.09	0.72		0.57
	14	9.28	0.63		0.48
	15	9.49	0.57		0.42
	16	9.77	0.43	0.16	0.27
	17	11.29	2.23		2.07

were added to each of the remaining duplicate tubes containing the buffer solutions. Lipase determinations were then carried out on these samples in the usual manner. Blank determinations, using buffer numbers 1, 5, 10 and 16, checked within experimental error. The data for three representative experiments are shown in Table XII.

Figure 5 gives the curves obtained for these three milks--curves "A" and "C" are for individual milks from two cows while curve "B" is for a mixed sample of milk from the University of Alberta herd. The optimum pH for hydrolysis of tributyrin by the milk lipase is between pH 8.7 and 8.8 when determined



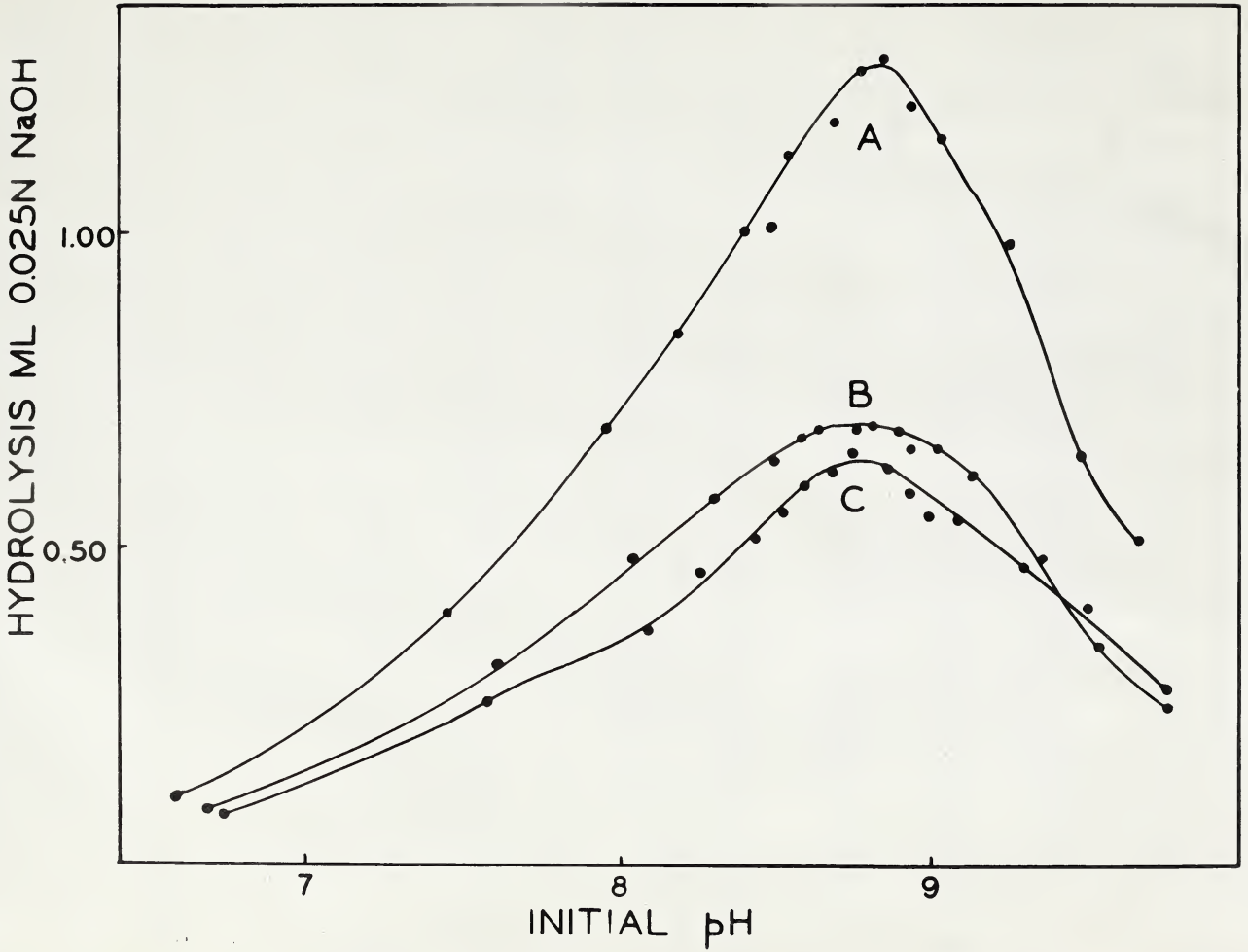


Figure 5 - Effect of Initial pH on Hydrolysis

at 30°C at the start of the reaction period. This pH optimum is slightly above that found by Mattick and Kay, and Peterson, Johnson and Price. However, these investigators do not mention the temperature at which they determined their pH values. The so-called "optimum pH" curve is actually a function of two variables:

(1) the stability of the enzyme protein to hydrogen ion concentration,

(2) the effect of hydrogen ion concentration on the catalyzed reaction.

As a result, the pH optimum may depend on a number of factors, such as substrate concentration, reaction time and temperature, nature of buffer and purity of enzyme preparation.

Saponification

The increased rate of hydrolysis at pH values above 9.75 is believed due to the increased saponification at high pH. In order to determine whether saponification at pH 8.75 could be a significant source of error in the lipase assay, determinations were carried out on buffered, pasteurized skim milk held at 37°C for increasing periods of time. Table XIII shows that over the 30 minute reaction period no significant saponification occurs.

pH Control by the Borate Buffer

Figure 5 shows that pH has a marked effect on the ability of the lipases in milk to hydrolize tributyrin and that

deviations from the optimum pH may be more important in the determinations where hydrolysis is greatest.

TABLE XIII

EFFECT OF REACTION TIME ON POSSIBLE SAPONIFICATION

Tributyrin Ml	pH at 30°C.	Reaction period Minutes	Titer
0.60	8.75	0	0.15
"	"	30	0.15
"	"	60	0.17
"	"	90	0.20

In the early stages of this investigation, various sodium borate buffers were tried in an attempt to find a suitable buffer that would give the desired initial pH with the milk sample and also have good buffer capacity without inhibiting the lipase enzyme. The following experiment illustrates the buffer capacity of the borate buffer suggested in this paper.

Eight milk samples from different cows in the University of Alberta herd were collected and held at 4°C for eight hours. Ten ml of each milk were buffered with 25 ml of the borate buffer. Quadruplicate test tubes containing 7 ml of each buffered milk were then prepared. The pH of one of the quadruplicate samples, to which 0.6 ml tributyrin had been added, was determined at 30°C before the reaction period.

The pH at the end of the 30 minute reaction period was also determined at 30°C, using the second of the quadruplicate tubes. In this case the pH was determined as quickly as possible after rapidly cooling the samples in a water bath from 37°C to 30°C. Lipase determinations were carried out on the remaining two tubes, one of the samples serving as the blank.

TABLE XIV

BUFFER CAPACITY OF SODIUM BORATE BUFFER

Sample No.	Initial pH 30°C	Final pH 30°C	pH Difference	Hydrolysis
1	8.65	8.53	0.12	0.31
2	8.69	8.55	0.14	0.46
3	8.64	8.42	0.22	0.86
4	8.64	8.43	0.21	0.79
5	8.70	8.56	0.14	0.61
6	8.78	8.59	0.19	0.60
7	8.75	8.35	0.40	1.37
8	8.74	8.44	0.30	1.08

Table XIV shows that the pH values of the buffered milks did not drop over 0.30 pH units except in the case of Sample 7 which had a high hydrolysis value. The milk from this

particular cow also gave the unusually high results discussed under "(a) Tributyrin Concentration." The first four samples of milk were from Jersey cows and gave slightly lower initial pH values. However, for routine determinations on fresh normal milks, few samples should require adjustment of pH. Where extensive lipolysis of the milk fat has occurred or where lactic acid has been produced, adjustment of pH may be necessary.

pH at Time of Extraction.

In order to stop hydrolysis of tributyrin and to facilitate the subsequent extraction of the butyric acid, 5 ml of M phosphoric acid are added to the sample after the reaction period. At the above stage in the lipase assay, pH determinations were made on the eight samples used in the above experiment and the results were found to be between pH 1.5 and 2.0.

(c) Optimum Reaction Period Temperature.

During the course of this investigation experiments were conducted to determine the optimum temperature for the hydrolysis of tributyrin during 30 and 60 minute reaction periods. The following experiment is representative of this work.

A sample of mixed milk was collected from the University farm herd and held approximately 5 hours at 4°C. Twenty ml

of skim milk from the above sample were added to 50 ml of borate buffer. Replicate 7 ml samples of the buffered milk were then prepared. Triplicate tubes were placed in water baths held at 30°, 37°, 40° and 45°C and allowed to reach the respective bath temperature in three minutes. One sample from each triplicate set served as a blank determination. Lipase estimations were made on the remaining duplicates using a 30 minute reaction period. A similar experiment was then carried out using milk from an individual cow from the University herd. The data are given in Table XV.

Figure 6 shows that, under the above conditions of the proposed lipase assay 37°C gave the greatest hydrolysis.

Mattick and Kay used a 6 hour incubation period and a temperature of 37°C, while Peterson, Johnson and Price suggested a 30 minute reaction period at 40°C. These workers did not give any data regarding the effect of reaction temperature on hydrolysis.

The temperature-activity curve of an enzyme can be resolved into two components:

(1) the effect of the increase in temperature on the rate of the catalyzed reaction.

(2) the effect of the increase in temperature on the stability of the enzyme.

Until further work could be done on the kinetics of the lipolytic enzymes of milk, a 30 minute reaction period at a temperature of 37°C was chosen for the proposed method.

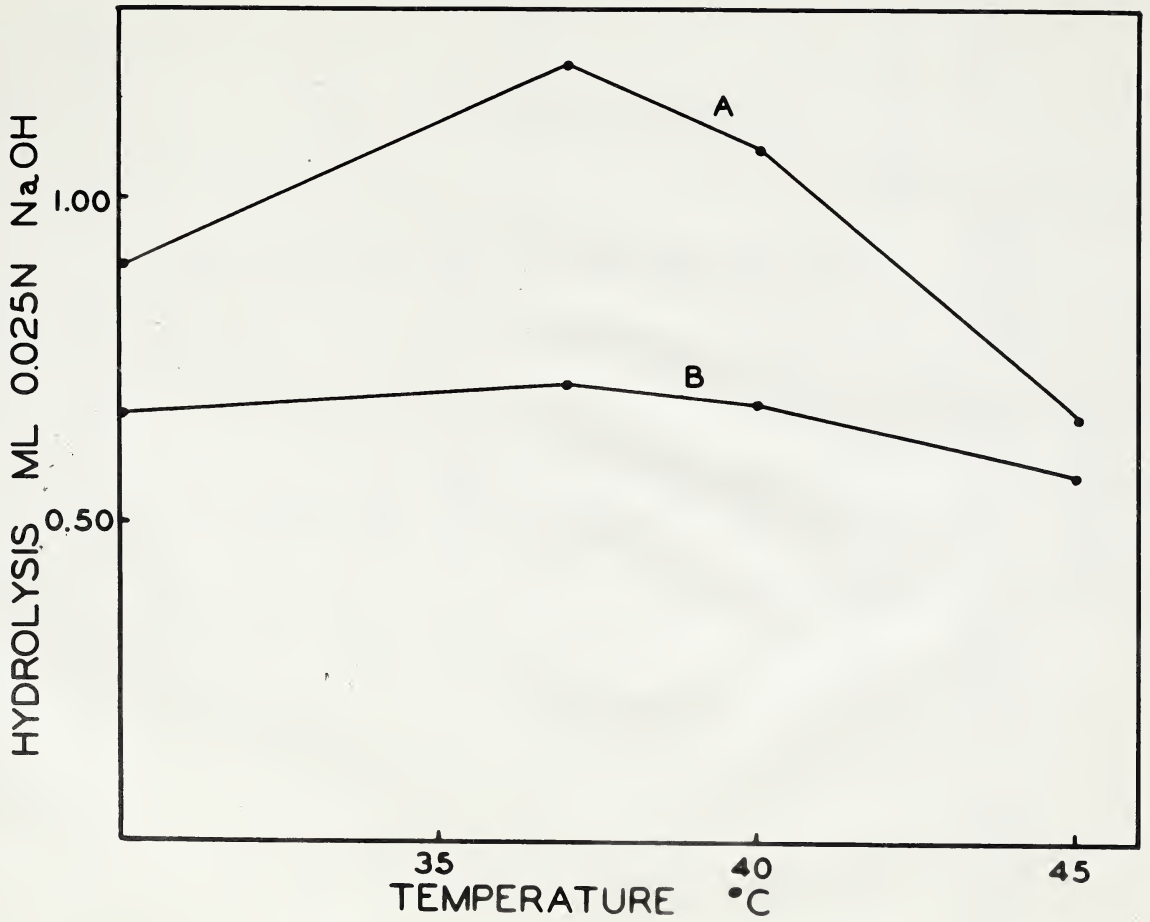


Figure 6 - Effect of Reaction Temperature on Hydrolysis

TABLE XV

OPTIMUM REACTION PERIOD

TEMPERATURE

Sample	Reaction Temp. °C	Titer	Blank	Average Hydrolysis
A-Mixed Milk				
	30	1.06	0.16	0.91
	30	1.08		
	37	1.37		1.22
	37	1.38		
	40	1.22		1.09
	40	1.28		
	45	0.81		0.67
	45	0.81		
B-Individual Milk				
	30	0.82	0.16	0.67
	30	0.84		
	37	0.86		0.71
	37	0.87		
	40	0.83		0.68
	40	0.85		
	45	0.72		0.57
	45	0.74		

However, it is recognized that such a reaction period temperature may be close to the point where little change in conditions with respect to pH, concentration of enzyme, substrate and products of the reaction, might tend to shift the reaction over to one of inactivation.

(d) Enzyme Concentration.

Preliminary experiments showed that varying amounts of milk could be added to selected buffers, whose composition is given in Table XI, to give similar pH values. The buffer solutions were all 0.1M with respect to boric acid but differed in concentration of NaOH present. The milks used in the following experiment had been held for varying periods of time from 8 to 24 hours at 4°C. Duplicate tubes containing 5 ml of each selected buffer were prepared. Increasing amounts of skim milk were added to one set of buffers and the pH determined at 22°C. The pH of the buffered milks of each series checked within ±0.04 pH units. The corresponding amounts of milk were then added to the duplicate set of test tubes containing 5 ml of each buffer. Both series of samples were brought up to 37°C. The first set, on which the pH values had been checked, served as blank determinations. The lipase activity of the second set was determined in the usual manner. Table XVI summarizes the data obtained for three different milks.

Figure 7 shows that there appears to be a direct relation between the enzyme concentration and hydrolysis of tributyrin when 0.50 to 2.00 ml of milk are used. Under the above conditions the concentration of enzyme present is the limiting factor in the hydrolysis of the substrate.

TABLE XVI

EFFECT OF ENZYME CONCENTRATION

ON HYDROLYSIS OF TRIBUTYRIN

	Milk Ml	Buffer Number	pH at 22°C.	Titer	Blank	Hydrolysis
Milk A						
	0.00	5	8.93	0.15	0.14	0.01
	0.10	5	8.89	0.25	0.14	0.11
	0.30	6	8.94	0.41	0.14	0.27
	0.50	6	8.93	0.55	0.15	0.40
	1.00	7	8.92	0.73	0.15	0.58
	1.50	8	8.91	1.00	0.15	0.85
	2.00	9	8.92	1.26	0.16	1.10
Milk B						
	0.00	5	8.87	0.10	0.13	0.00
	0.50	6	8.87	0.43	0.15	0.28
	1.00	7	8.87	0.57	0.15	0.42
	1.50	8	8.85	0.81	0.16	0.65
	2.00	9	8.86	0.97	0.16	0.81
	2.50	10	8.88	1.16	0.17(Est.)	0.99
Milk C						
	0.00	5	8.87	0.13	0.12	0.01
	0.50	6	8.88	0.39	0.14	0.25
	1.00	7	8.88	0.56	0.15	0.41
	1.50	7	8.83	0.70	0.15	0.55
	2.00	9	8.91	0.86	0.15	0.71

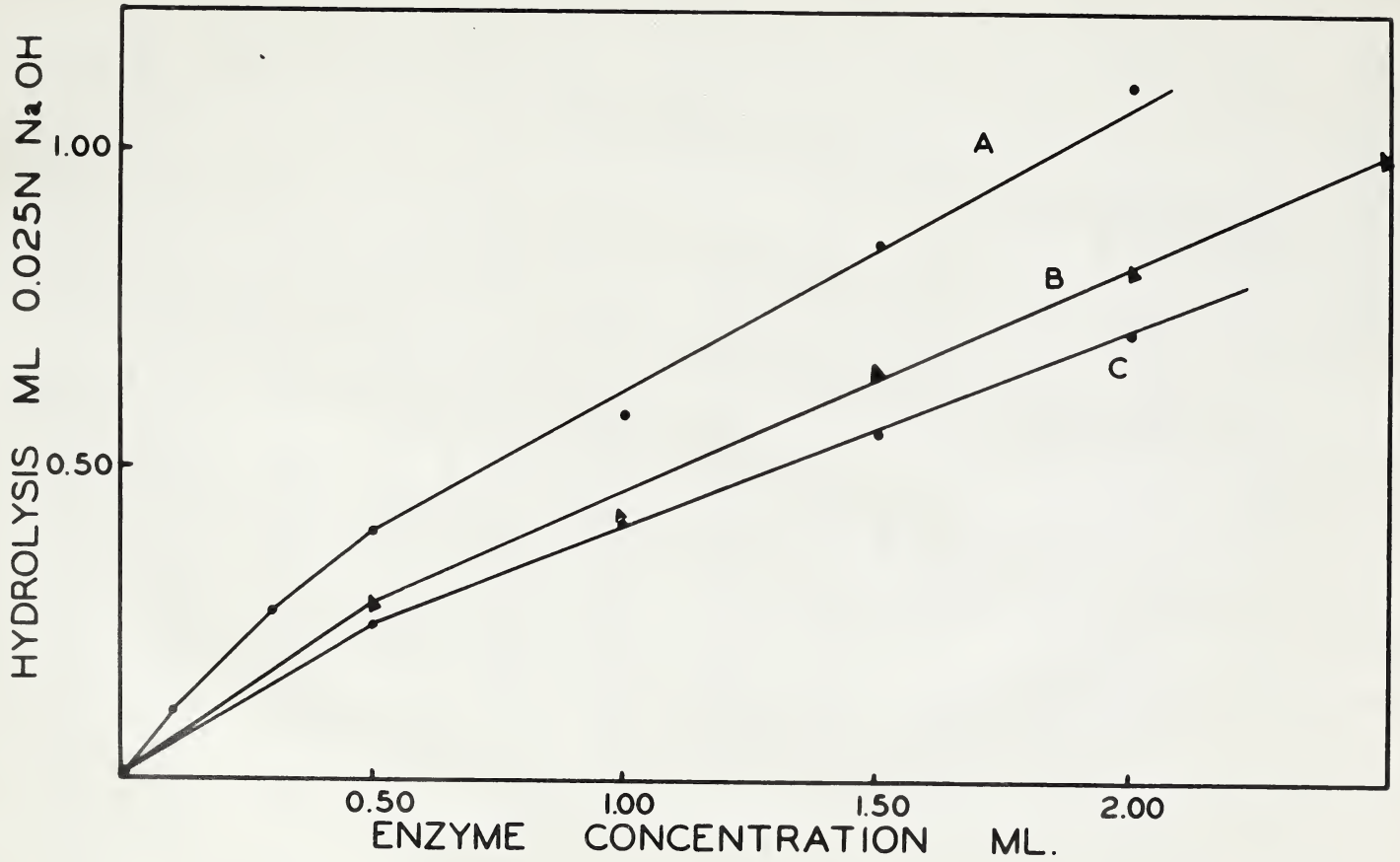


Figure 7 - Effect of Enzyme Concentration on Hydrolysis

(e) Reaction Time

Several experiments were carried out in the course of this investigation to determine the effect of the reaction time on hydrolysis. In one such experiment two samples of milk, one from a Holstein cow and one from a Jersey cow, were obtained from the University herd and held at 4°C for 6 hours. Thirty ml of each milk were buffered with 75 ml of borate buffer and the pH adjusted to 8.8 at 22°C. Seven ml of each buffered milk were then pipetted into replicate test tubes. Tributyrin was added to each sample tube and the determinations started in the usual manner. Duplicate tubes were removed from the 37°C water bath at increasing intervals of time, M phosphoric acid added and the determinations completed.

Fifty ml of each milk were heated to 90°C, held two minutes at this temperature and then quickly cooled to 25°C. Reaction mixtures consisting of 2 ml milk, 5 ml buffer and 0.6 ml tributyrin were prepared using the above milks and these were held 0, 30, 60 and 90 minutes. These determinations served as blanks to show the possible breakdown of tributyrin in the absence of lipase activity. The data obtained are given in Table XVII.

The curves shown in Figure 8 indicate that the rate of hydrolysis was quite constant for the first 20 minutes of the reaction period and then it decreased appreciably.

TABLE XVII

EFFECT OF REACTION TIME ON HYDROLYSIS

Milk	Reaction Time, Min.	Titer	Blank	Average Hydrolysis
A	0	0.16	0.15	0.02
	0	0.17		
	10	0.58		0.44
	10	0.61		
	15	0.76	0.15	0.59
	15	0.72		
	20	0.98		0.84
	20	1.00		
	30	1.32	0.16	1.16
	30	1.32		
	45	1.71		1.54
	45	1.69		
	60	2.00	0.17	1.83
	60	1.99		
	90	2.72	0.19	2.56
	90	2.77		
B1	0	0.16	0.15	0.01
	0	0.15		
	10	0.46		0.29
	10	0.44		
	20	0.73		0.59
	20	0.75		
	30	0.96	0.15	0.82
	30	0.97		
	45	1.30		1.13
	45	1.27		
	60	1.60	0.17	1.40
	60	1.53		
	90	2.05	0.20	1.89
	90	2.11		

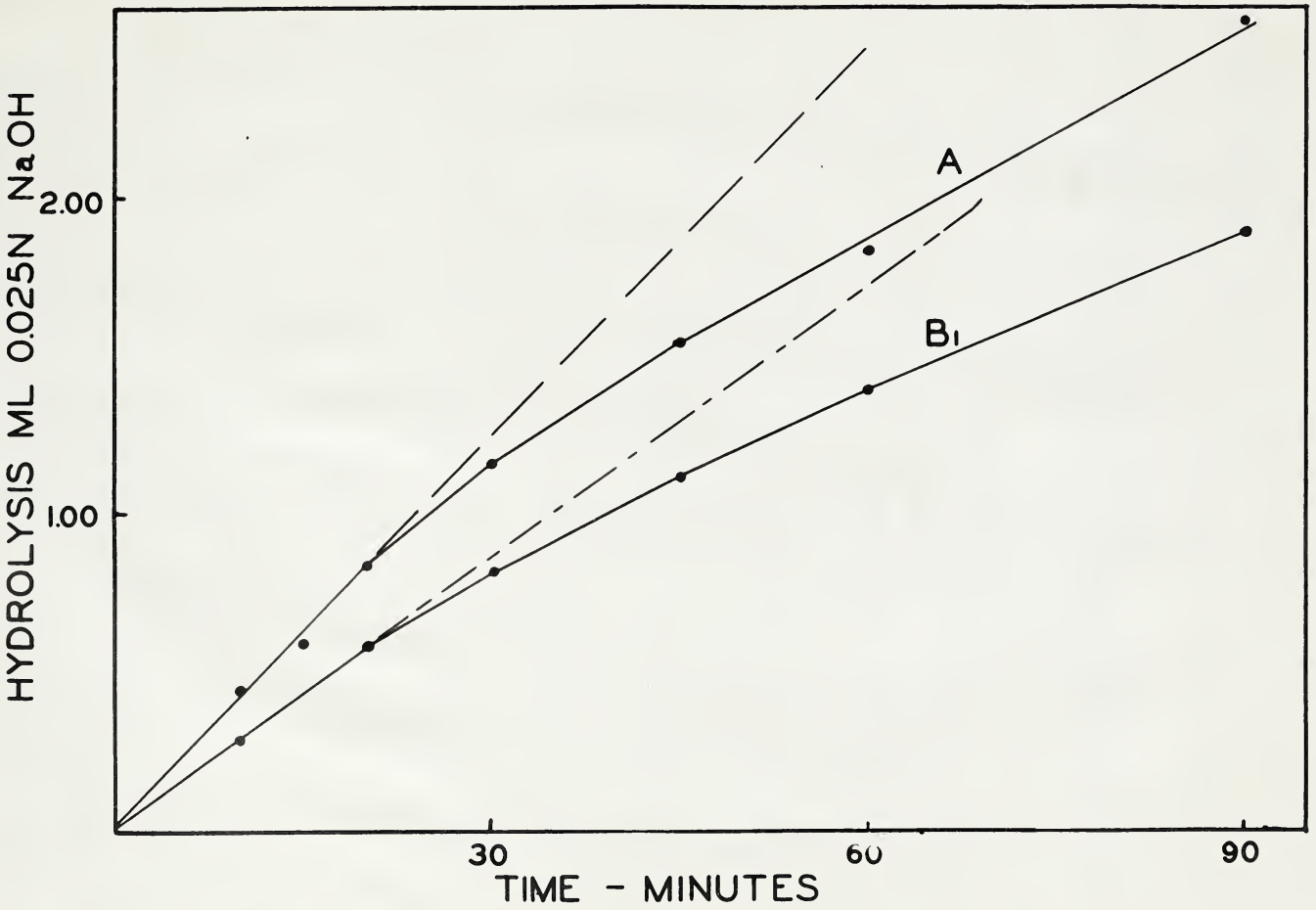


Figure 8 - Effect of Reaction Time on Hydrolysis

This decrease may be due to various factors such as:

(1) a partial inactivation of the enzyme by the 30 minute reaction time at 37°C.

(2) a decrease in pH during the reaction period may affect the activity of the enzyme.

(3) the accumulation of the products of the reaction may inhibit the enzyme.

(4) the presence of impurities.

With a 30 minute reaction period the percentage decrease in hydrolysis was 6 and 7 percent for milks A and B1 respectively. Using the 60 minute reaction period, the corresponding percentage decreases were 19 and 24 percent respectively. In order to obtain significant hydrolysis without a large deviation from the expected value, the 30 minute reaction period was chosen for the lipase assay.

Since a temperature of 40°C for 30 minutes results in a decrease in lipase activity as shown in "(c) Optimum Reaction Period Temperature," further work may show the advisability of lowering the reaction temperature below 37°C. While it has been found that the borate buffer used in the determination will keep the difference in the pH of the reaction mixture at the beginning and end of the reaction time within 0.4 pH units, an improved buffer might prevent this drop in pH. The effect of the accumulating free butyric acid, which is in the form of highly ionized sodium salt, should also be investigated. It may be possible to fractionate milk in

order to obtain a relatively pure enzyme preparation. Such a preparation would be very useful in further study of the kinetics of the enzyme.

(f) $\underline{CaCl_2}$

Peterson, Johnson and Price found that zinc chloride, potassium cyanide, manganous sulfate, magnesium chloride and cysteine did not appear to activate milk lipase. However, Goldstein and Roe (1943) used calcium acetate in their technique for the determination of the lipase of blood serum.

Early in this work it was decided to investigate the effect of adding $CaCl_2$ to the reaction mixture as an activator. Preliminary investigation indicated that $CaCl_2$ appeared to have a marked accelerating effect on the hydrolysis of tributyrin by milk lipase. Several experiments were then carried out to find the concentration of $CaCl_2$ required for optimum hydrolysis under the conditions of the proposed lipase assay. The results of one such study are reported here.

Mixed Jersey milk from the University of Alberta herd was held approximately 24 hours at 4°C. Sixty ml of this milk (skimmed) were buffered with borate buffer and replicate tubes, each containing 7 ml of the mixture, were prepared. The samples were brought up to 37°C and increasing amounts of 10% $CaCl_2$ were added to duplicate tubes. After adding 0.20 ml tributyrin, the determinations were completed in the usual manner. Blank determinations using 0.00, 0.25 and

0.50 ml of 10% $CaCl_2$ were made and these checked within experimental error.

Figure 9 shows that, under the conditions of this particular experiment, maximum hydrolysis occurred when 0.20 to 0.30 ml of 10% $CaCl_2$ were added to the reaction mixture. When the amount of $CaCl_2$ was increased over 0.30 ml, hydrolysis was decreased.

It was decided to study further the use of 0.25 ml of $CaCl_2$ as an activator in the proposed lipase assay. Using this modification of the procedure, the following factors were investigated:

(1) Optimum pH at start of Reaction Period for Hydrolysis.

(2) Effect of Tributyrin on Hydrolysis.

(3) Effect of Reaction Temperature on Hydrolysis.

(4) Effect of Heat on Lipase of Milk.

(5) Effect of Storage Temperature of the Milk on Lipase.

(6) Effect of Shaking during the Reaction Period on Hydrolysis.

The data obtained for the above are not reported in this paper since it was later decided not to use $CaCl_2$ in the lipase determination.

Several experiments were also carried out on the effect of $CaCl_2$ on milks obtained from different cows. In one representative experiment, samples of milk from five Holstein and five Jersey cows were collected at the University of Alberta Farm and held approximately one hour at 4°C. Lipase

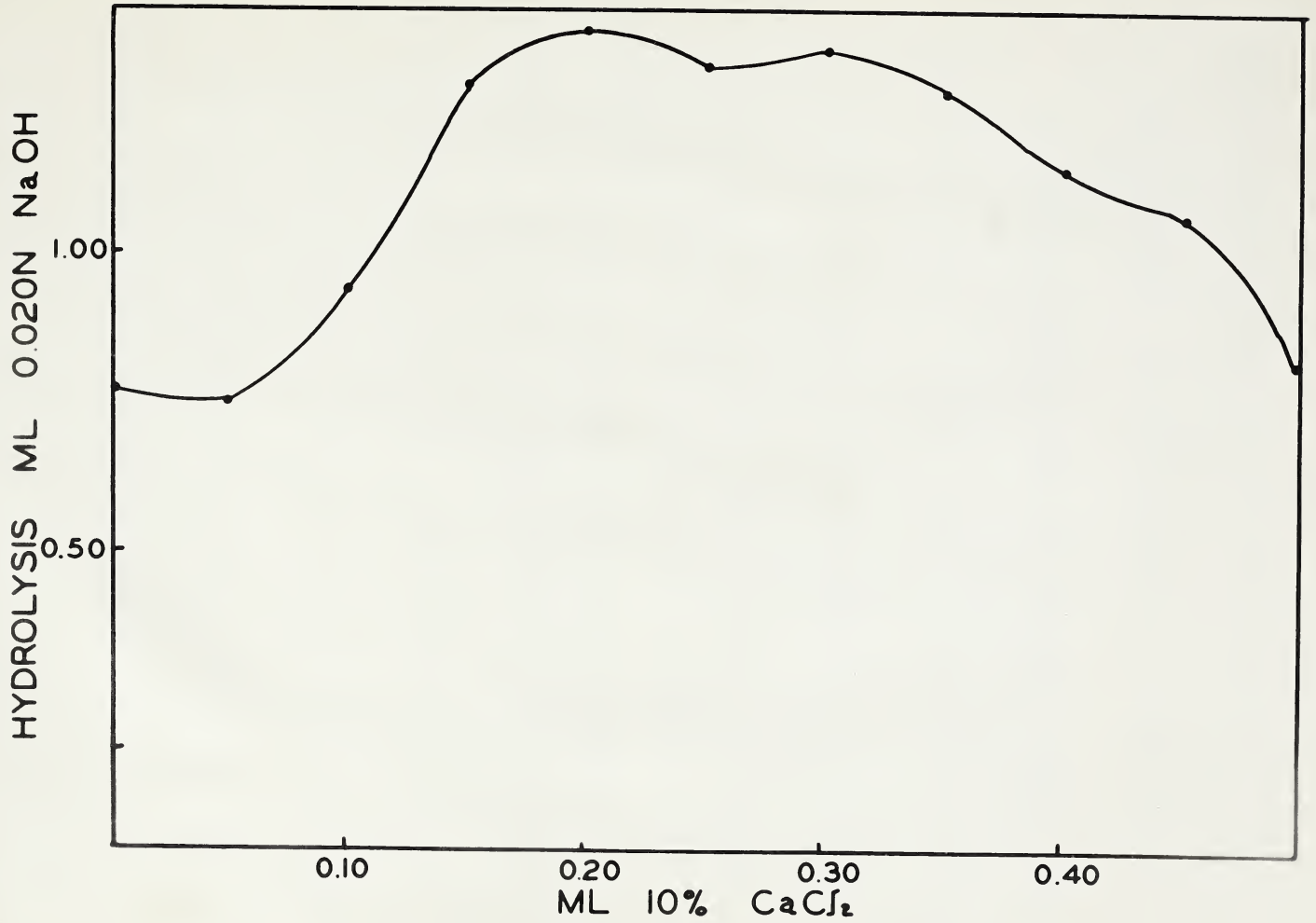


Figure 9 - Effect of $CaCl_2$ on Hydrolysis

determinations including the addition of 0.25 ml of 10% $CaCl_2$ were compared with determinations made at the same time where no $CaCl_2$ was used. The data obtained are summarized in Table XVIII.

The results indicate that the addition of $CaCl_2$ seemed to increase the hydrolysis of tributyrin by each milk. Since NaCl did not give a similar effect, it seems possible that the Ca++ ion may be responsible. The actual manner in which the Ca++ ion may bring about the activation is not known. However, it may possibly prevent the accumulation of free butyric acid at the enzyme "active centers" by combining with the butyric acid as soon as it is produced from the substrate.

The relative activation varied with the different milk samples. The amount of $CaCl_2$ used (0.25 ml) may not have been the concentration required to give optimum activation with each milk. Since there are differences in the salt composition of individual milks, it may be impossible to use the same amount of $CaCl_2$ and obtain optimum activation in each case. Therefore, it was decided not to use $CaCl_2$ in the routine determination of lipase until more data were obtained to show the precise amount required to give optimum hydrolysis with different milks.

TABLE XVIII

EFFECT OF $CaCl_2$ ON HYDROLYSIS OF TRIBUTYRIN BY DIFFERENT MILKS

Sample Number	10% $CaCl_2$ Ml	Average Titer	Blank	Hydrolysis Ml 0.020N NaOH	% Increase Hydrolysis
1	0.00	0.86	0.15	0.71	56
1	0.25	1.26	0.15	1.11	
2	0.00	1.33	0.15	1.18	75
2	0.25	2.22	0.16	2.06	
3	0.00	1.19	0.15	1.04	52
3	0.25	1.74	0.16	1.58	
4	0.00	0.89	0.16	0.73	52
4	0.25	1.27	0.16	1.11	
5	0.00	1.07	0.15	0.92	77
5	0.25	1.80	0.17	1.63	
6	0.00	1.00	0.16	0.84	65
6	0.25	1.57	0.18	1.39	
7	0.00	0.94	0.16	0.78	54
7	0.25	1.36	0.16	1.20	
8	0.00	0.93	0.15	0.78	18
8	0.25	1.08	0.16	0.92	
9	0.00	1.98	0.16	1.82	42
9	0.25	2.75	0.16	2.59	
10	0.00	1.12	0.15	0.97	43
10	0.25	1.55	0.16	1.39	

DISCUSSION

The term "lipase" has been used throughout this paper with reference to the enzyme in milk responsible for the hydrolysis of tributyrin. In the literature the general term "esterase" has been commonly used as a group name for all enzymes catalyzing the hydrolysis of esters whether the latter are simple or complex. The use of the term "lipase" has been restricted to those enzyme preparations whose major specificity is toward glyceride substrates. While tributyrin is a triglyceride, it is probably not a naturally occurring fat. Mattick and Kay preferred to refer to the milk enzyme as a "tributyrinase." It seems reasonable to surmise that the enzyme in milk responsible for hydrolysis of the milk fat, which is a mixture of triglycerides containing a wide variety of fatty acids, should be able to hydrolyze the tributyrin substrate. However, further work using other triglycerides as substrates is needed to confirm this point as well as the presence of more than one lipase. The comparative solubilities in ether of the acids produced from other substrates would have to be considered in such a study involving the use of the proposed lipase assay.

The method as described in this paper provides a convenient routine test for studies on lipolysis and lipolytic activity of different milks and bacterial cultures. Dunkley

has applied a method similar in principle to investigate the correlation between the lipase content and lipolysis, as shown by surface tension changes, over the lactation period. Lubert has used a method based on this procedure to study lipolytic activity in cultures of various micro-organisms and in cheddar cheese.

It is believed that the proposed method could be used to investigate the following factors in relation to the lipase activity of milk:

(1) Heat, including the "temperature activation" treatment investigated by Krukovsky and Herrington.

(2) Stability of milk lipase in relation to storage time, temperature and pH.

(3) Homogenization and drying.

(4) Light.

(5) Oxidation-reduction potentials.

(6) Metals.

(7) Activators and inhibitors.

(8) Distribution of the enzyme between the fat and aqueous phase of milk.

(9) Correlation between blood serum lipase and milk lipase during the lactation period.

A detailed study of the kinetics of milk lipase would be of theoretical and possibly practical interest. However, it might be necessary to prepare a purified or partially purified enzyme preparation for such an investigation.

SUMMARY AND CONCLUSIONS

1. A method for the determination of the lipase content of or lipolytic activity in milk has been presented. The procedure involves the ether extraction of the butyric acid released by the hydrolysis of the tributyrin substrate under standardized conditions in the presence of the milk enzyme. The lipase activity is expressed as ml of 0.025N NaOH required to neutralize a 5 ml aliquot of the ether extract.

2. Experimental data have been offered to show that, under the conditions of the lipase assay, the ether extraction procedure is a reliable measure of the butyric acid produced from the substrate.

3. Various factors affecting the blank determination have been studied.

4. Several other factors affecting the method have been investigated.

In the presence of excess substrate (tributyrin) the enzyme followed a zero order reaction.

Under the experimental conditions used, the apparent optimum reaction temperature and pH were $37^{\circ}C$ and pH 8.75.

There was a direct relation between the enzyme concentration and the hydrolysis of tributyrin.

The rate of hydrolysis decreased after the first 20 minutes of the reaction period.

The hydrolysis of tributyrin by milk lipase was increased appreciably by the presence of $CaCl_2$.

5. It should be possible to use modifications of the procedure to study lipase activity in dairy products and in bacterial cultures.

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